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Functional conservation of the hydrophobic domain of polypeptide 3AB between human rhinovirus and poliovirus

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Abstract

In this study we exchanged portions of the poliovirus type 1 (PV1) hydrophobic domain within the membrane-associated polypeptide 3AB for the analogous sequences from human rhinovirus 14 (HRV14). The sequence exchanges were based upon a previous report in which the 22 amino acid hydrophobic region was subdivided into two domains, I and II, the latter of which was shown to be required for membrane association (J. Biol. Chem. 271 (1996), 26810). Using these divisions, the HRV14 sequences were cloned into the complete poliovirus type 1 cDNA sequence. RNAs transcribed from these cDNAs were transfected into HeLa cell monolayers and used in HeLa cell-free translation/replication assays. The data indicated that 3AB sequences from PV1 and HRV14 are interchangeable; however, the substitutions cause a range of significant RNA replication defects, and in some cases, protein processing defects. Following transfection of RNAs encoding the domain substitutions into HeLa cell monolayers, virus isolates were harvested, and the corresponding viral RNAs were sequenced. The sequence data revealed that for the carboxy-terminal domain substitutions (domain II), multiple nucleotide changes were identified in the first, second, and third positions of different codons. In addition, the data indicated that for one of the PV1/HRV14 chimeras to replicate, compensatory mutations within poliovirus protein 2B may be required.

Introduction

Similar to most positive-strand RNA viruses, picornaviruses amplify their genomic RNA in the context of membrane-associated replication complexes (Caliguiri and Tamm, 1969; Caliguiri and Tamm, 1970a, 1970b; Bienz et al., 1992, 1994). All newly synthesized RNA chains have a small, viral-encoded peptide termed VPg (or 3B) covalently attached to their 5′ termini via a phosphodiester-like linkage (Lee et al., 1977; Flanegan et al., 1977; Rothberg et al., 1978; Ambros and Baltimore, 1978). This protein–RNA linkage is thought to be the result of a protein-priming mechanism in which a uridylylated form of VPg serves as a protein primer for the RNA-dependent RNA polymerase ($3D^{pol}$) to initiate RNA synthesis (Nomoto et al., 1977; Pettersson et al., 1978; Takegami et al., 1983; Paul et al., 1998). It has been shown that the uridylylation event is dependent, in part, upon an internal *cis*-acting RNA element (*cre*), an element first reported for human rhinovirus (McKnight and Lemon, 1996, 1998) and subsequently found in a number of other picornavirus genomes (Lobert et al., 1999; Goodfellow et al., 2000; Paul et al., 2000; Gerber et al., 2001; Mason et al., 2002). The presence of this internal RNA hairpin appears to be essential for viral RNA synthesis (Rieder et al., 2000; Paul et al., 2000; Yang et al., 2002; Goodfellow et al., 2003).

VPg is a strongly basic protein (pI > 10) that is utilized in a hydrophobic environment. This has led to the hypothesis that VPg is delivered to the replication machinery as part of a larger lipophilic protein that contains the VPg sequences (Semler et al., 1982). One candidate for such a viral protein is poliovirus polypeptide 3AB, which contains the VPg sequence in addition to a 22 amino acid stretch of nonpolar residues. It was previously shown that poliovirus

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3AB associates with microsomal membranes in a manner consistent with that of an integral membrane protein (Datta and Dasgupta, 1994; Towner et al., 1996). Since the 22 amino acid hydrophobic domain is highly conserved among various members of the enterovirus and rhinovirus genera (Kitamura et al., 1981; Racaniello and Baltimore, 1981; Stanway et al., 1984; Giachetti and Semler, 1990; Towner et al., 1996), it is reasonable to predict a common mechanism for VPg delivery to the replication complex. Indeed, the idea of functional conservation within the replication machinery among members of the rhinovirus and enterovirus genera has been recently supported by the ability to isolate virus from chimeric poliovirus RNA possessing a VPg from human rhinovirus 14 (HRV14), and furthermore, that the HRV14 VPg can be uridylylated in vitro by poliovirus $3D^{pol}$ (Paul et al., 2003).

The 22 amino acid nonpolar sequence in 3A, referred to as the hydrophobic domain, has been further subdivided into two smaller domains, domains I and II, in which the hydrophobicity present in domain II was found to be essential for 3AB membrane association (Towner et al., 1996). Interestingly, the sequences in domain I could be completely removed with no apparent effect on membrane association. Yet single amino acid substitutions of conserved residues within the same domain result in viruses with severe RNA replication defects, particularly at the level of initiation of positive-strand RNA synthesis (Giachetti and Semler, 1991). In addition, single and multiple amino acid substitutions of nonconserved residues in domain I or II can result in significant in vitro protein processing defects characterized by the overproduction of proteins P2 and 3BCD and the underproduction of protein 2C (Giachetti et al., 1992). Taken together, these results suggest that the two adjacent domains within the hydrophobic region may be involved in multiple structural support functions, effector domains, or protein subunit interfaces.

There is evidence that the function(s) of the hydrophobic domain is not completely conserved among the entero- and rhinovirus members because multiple poliovirus and rhinovirus mutants that have developed tolerance to the drug enviroxime contain mutations within 3AB (or 3A, the same polypeptide without VPg amino acid sequences at its carboxy-terminus) that are consistently clustered in different regions of the protein. The drug tolerant mutations in HRV14 tend to involve charged residues in the middle of 3AB, while the analogous phenotypic changes in poliovirus are consistently clustered within the hydrophobic domain (Heinz and Vance, 1995, 1996). Therefore, to determine if the entire hydrophobic domain, or only a portion thereof, is functionally conserved among members of the entero- and rhinovirus genera, the analogous sequences within the HRV14 hydrophobic domain were substituted in multiple combinations for the corresponding sequences within poliovirus protein 3AB. While the overall hydrophobicity of the 22 amino acid hydrophobic domain is well conserved, the identity at the amino acid level is less than 50%. These chimeric PV1/HRV14 3AB sequences were then placed in the poliovirus type 1 background and assayed for infectivity as well as RNA synthesis in both HeLa cells and in a poliovirus cell-free translation/replication assay. In this study, we report that the HRV 14 domain I substitution within 3AB results in an infectious RNA, while those chimeras containing domain II substitutions are quasi-infectious. Multiple reversion mutants were isolated, including one with a second site lesion in poliovirus protein 2B.

Results

In vitro translation and RNA replication

As an initial assessment of RNA synthesis and protein processing in the absence of the multiple rounds of genetic selection and amplification that take place in infected cells, each of the full-length RNAs harboring the domain substitutions (Fig. 1) was translated and replicated in a HeLa cell-free translation/replication assay. The results of this analysis are displayed in Fig. 2. Fig. 2A shows the translation and protein processing directed by each RNA, while Fig. 2B shows the corresponding in vitro RNA replication assays. As shown in lanes 3 and 5 of Fig. 2A, a slight defect in protein processing can be seen when compared to that of wild-type for PV RNAs containing the HRV14 domain I or domain I+II sequences. This defect is characterized by a small increase in 3BCD and corresponding decrease in $3D^{pol}$. Compared to wild-type, the substitution of HRV14 domain II sequences does not result in any protein processing defect (compare lane 4 to lane 2). In all cases, the domain substitutions confer significant electrophoretic mobility differences upon the 3AB protein compared to wild-type.

When the level of primary RNA replication by each of the mutants was measured in vitro, significant differences among them were evident. As shown in Fig. 2B, lane 4, the substitution of the domain I sequences results in a significant RNA replication defect compared to wild-type (lane 3) at the level of synthesis of both single-stranded and replicative intermediate/replicative form RNAs. This defect in RNA synthesis by DIS may be due to the protein processing defect (a processing defect potentially causing decreased levels of $3D^{pol}$). Interestingly, the DIIS RNA, which directs wild-type-like protein processing, replicates at even lower levels than the DIS RNA, indicating that the HRV14 domain II sequences likely confer a true RNA synthesis defect. Finally, as shown in Fig. 2B (lane 6), substitution of the entire HRV14 hydrophobic domain, except for the first five amino acids at the N-terminal portion, results in a dramatic RNA replication defect. The defect in RNA replication may be due, in part, to the above-described protein processing defect. It is noteworthy that little 3AB can be seen in lane 5 of Fig. 2A, although it can be seen after long exposures of the autoradiograph (data not shown).

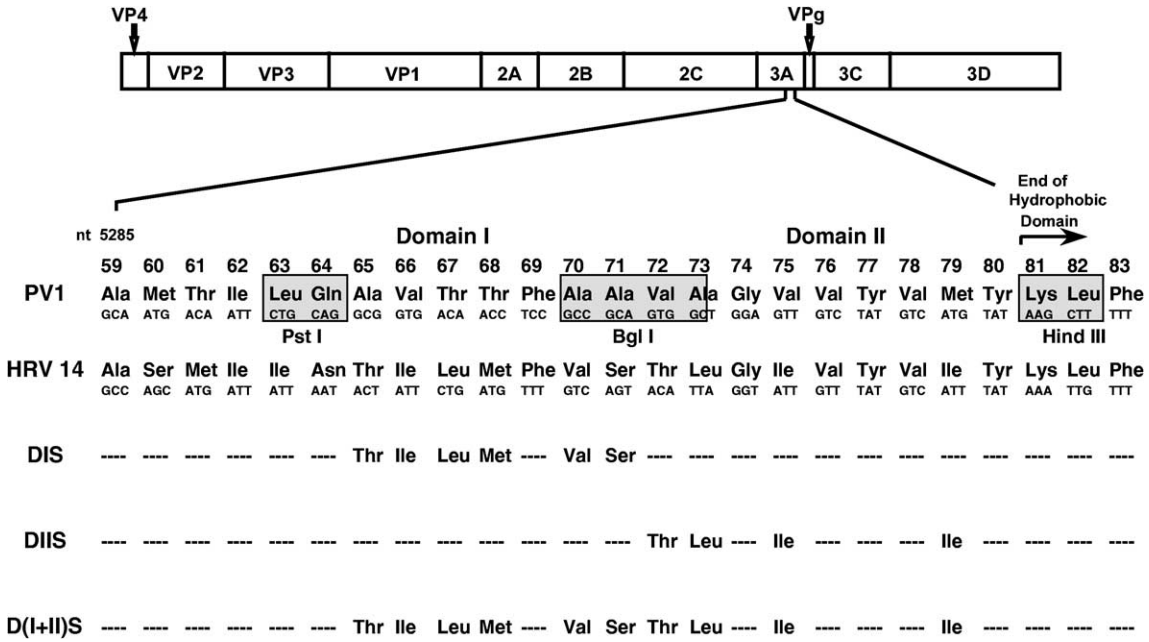

Fig. 1. Nucleotide sequences and amino acid substitutions of the hydrophobic domain of 3AB. The nucleotide sequences (as cDNA) and amino acids of the poliovirus hydrophobic domain are aligned with the analogous sequences of human rhinovirus. Below each of the PV1 and HRV14 sequences are the amino acid substitutions in which DIS indicates the substitution of HRV14 sequences for those of PV1 between the *Pst*I and *Bgl*I sites (domain I), while DIIS indicates the substitution of the analogous HRV sequences for those of PVI between the *Bgl*I and *Hin*dIII sites (domain II). D(I+II)S indicates the substitution of the HRV sequences between the *Pst*I and *Hin*dIII sites (domains I+II). Boxed regions in gray outline the restriction sites used for the mutagenesis where both the *Pst*I and the *Hin*dIII sites contain genetically engineered silent mutations to create the restriction site (Giachetti and Semler, 1991, 1992). For all substitutions listed, the complete HRV 14 nucleotide sequences were used to encode the indicated amino acid changes, including silent mutations.

Transfection of HeLa cells and plaque isolation

To determine if the HRV14 sequence substitutions into the PV 3AB hydrophobic domain (outlined in Fig. 1) result in the generation of infectious viruses, transcribed RNAs corresponding to wild-type PV1 and each domain substitution were separately transfected into HeLa cell monolayers at 33 and 37°C. Each RNA was transfected in duplicate at the concentrations of 5, 0.5, 0.05, and 0.005 μg per 60-mm-diameter dish and overlaid with semisolid or liquid media. At 48, 72, 96, and 120 h after transfection, the plates were examined for cytopathic effect (CPE), plaque size, and plaque number. The results of the RNA transfection experiment are summarized in Table 1. RNAs encoding the domain I substitution had a specific infectivity similar to that of wild-type RNA as evidenced by the appearance of equal numbers of plaques per microgram of RNA. At 37°C, the plaques generated from the DIS RNA were heterogeneous in size but were consistently smaller than those of wild-type poliovirus. In contrast, transfection of both the DIIS and the D(I+II)S RNAs resulted in 100- to 1000-fold fewer plaques per microgram of RNA compared to that of either wild-type or DIS. Nearly identical results were seen for those transfection assays carried out at 33°C with the exception that the overall growth kinetics for wild-type PV1 and all three domain substitutions were delayed by approximately 12–24 h (data not shown). Based upon the similar transfection results for each RNA at 33 C versus 37°C, only plaques isolated at 37°C were chosen for expansion and further analysis. Primary plaques present on the wild-type and DIS plates at 37°C were harvested at 48 and 72 h, while the plates for both DIIS and D(I+II)S were incubated for an additional 24 to 48 h prior to plaque isolation. For each domain substitution, three primary plaques were used for a second round of plaque purification.

Primary transfections in which the monolayers were overlaid with liquid media were carried out three separate times with each experiment yielding consistent times for the onset of CPE. In only one experiment were HeLa monolayers additionally overlaid with semisolid media for the purpose of harvesting primary plaques directly following the RNA transfection. A summary of the isolates harvested and time for the onset of CPE for each of the domain substitutions is presented in Table 2. Based upon these data, the domain I substitution appears to be infectious, while the domain II and domain I+II substitutions appear to be quasi-infectious.

Nucleotide sequences of recovered virus isolates

Following secondary plaque purifications, each plaque was used to generate passage 1 (P1) and passage 2 (P2) virus stocks. HeLa cell monolayers were infected with 0.5 ml of P1 stock, and total RNA was harvested at the time of onset of CPE. In contrast to the pronounced differences in the kinetics of appearance of CPE following transfection of

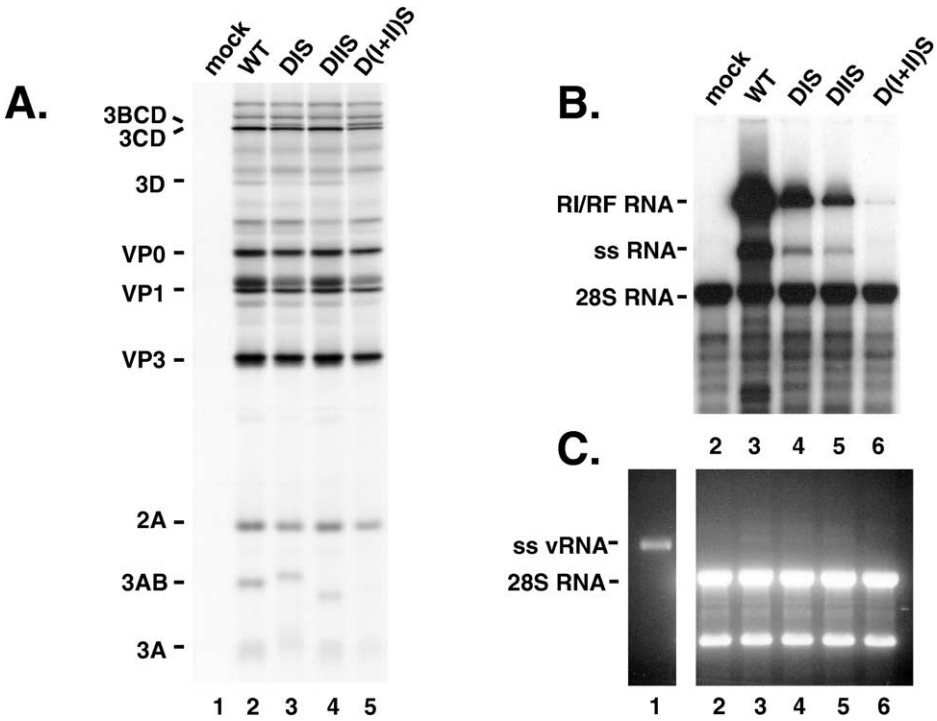

Fig. 2. Analysis of translation and replication properties using the coupled in vitro translation/replication assay. (A) shows the translation and protein processing profiles of each of the indicated RNAs. Proteins were labeled with [^{35}S]methionine. The reaction in lane 1 was not programmed with any PV RNA, while lanes 2 through 5 were programmed with 250 ng of each individual RNA (per 10 μl reaction). Half of each translation reaction was then subjected to electrophoresis on a 12.5% polyacrylamide–SDS gel and subsequent fluorography prior to exposure to film. (B) shows the corresponding in vitro RNA replication reactions in which RNAs synthesized in the cell-free extracts were labeled by incorporating [α^{32}P]CTP. The reaction in lane 2 was not programmed with any PV RNA, while lanes 3 through 6 were programmed with each of the respective RNAs. As a gel loading control, (C) shows the same gel as in (B) after staining with ethidium bromide to detect ribosomal RNAs (28S RNA) as well as virion RNA (ss vRNA) from poliovirus. In a separate experiment, PV1 RNA was translated and replicated in the presence of 2 mM guanidine HCl, in which PV translation proceeded unimpaired while RNA replication was completely inhibited (data not shown).

DIIS or D(I+II)S RNA, CPE on each infected plate appeared within 2–4 h of that for wild-type virus at 37°C, indicating that the DIIS and D(I+II)S mutants had most likely accumulated reversions or second site mutations that allowed for more rapid virus growth. Consistent with this possibility, P1 titers for all nine of the mutant virus stocks used for Northern blot analysis (see below) ranged from 1.6 $\times$ 10^8 to 7 $\times$ 10^8 PFU/ml. To initially confirm the maintenance of the HRV14 substitutions and to identify any reversions or mutations that may have accumulated, the virus population from infection by each isolate was sequenced within the 3AB hydrophobic domain using asymmetric RT-PCR followed by dideoxy sequencing. The results of the sequence analysis are presented in Fig. 3. Sequencing revealed consistency between members of a pair of isolates, indicating that the nucleotide changes were not the result of polymerase errors introduced during the reverse transcription or PCR amplification. Nucleotide changes that appeared ambiguous (representing possible sequencing artifacts or heterogeneity in the quasispecies population) were observed, but not listed. All of the amino acid changes shown were the result of single adenosine-to-guanosine transitions (per codon) except for the leucine-to-serine codon changes in DIIS isolates 6.1 and 6.2, which were transitions from uracil to cytosine.

For the DIS mutation, no complete reversions of any

Table 1
Summary of RNA transfections

Type of RNA	RNA transfected per 60-mm dish (μg)			
	5.0	0.5	0.05	0.005
Wild-type	All cells dead	All cells dead	>100 plaques	10–15 plaques
DIS	All cells dead	All cells dead	>100 plaques	5–10 plaques
DIIS	5–10 plaques	1–2 plaques	No plaques	No plaques
D(I+II)S	5–10 plaques	No plaques	No plaques	No plaques

Table 2
Summary of virus isolates containing 3AB hydrophobic domain substitutions

RNA	Primary isolate no.	Secondary isolate no.	Northern blot analysis
DIS (24 h)	1	1.1	Yes
		1.2	No
	2	2.1	No
		2.2	Yes
	3	3.1	Yes
		3.2	No
DIIS (48 h)	4	4.1	Yes
		4.2	No
	5	5.1	Yes
		5.2	No
	6	6.1	Yes
		6.2	No
D(I+II)S (72 h)	7	7.1	Yes
		7.2	No
	8	8.1	Yes
		8.2	No
	9	9.1	Yes
		9.2	No

Note. Three well-isolated plaques for each RNA were picked using a sterile Pasteur pipette and placed in MEM. The primary plaques were then diluted serially and used to infect HeLa monolayers at 37°C for the purpose of picking secondary plaques. Two secondary plaques were picked per primary isolate. One secondary isolate was then used to generate passage 1 and passage 2 virus stocks, which were subsequently used to infect HeLa cells to analyze the kinetics of RNA accumulation by Northern blot analysis. Shown in parentheses next to the name for each RNA is the approximate time for CPE to be observed in HeLa cells (with liquid culture overlays) transfected with 5 μg of the corresponding RNA.

nucleotides encoding the hydrophobic domain were seen (partial, ~50%, adenosine-to-guanosine transitions were seen for all six secondary isolates, a change that would revert the threonine 65 to the PV alanine), a result consistent with the high-specific infectivity of the RNA compared to that of DIIS or D(I+II)S.

Substitution of domains I+II of HRV14 into poliovirus resulted in RNA with the lowest specific infectivity. Sequence analysis revealed that all six isolates consistently contained at least one first position nucleotide change resulting in the reversion of threonine at position 65 to the PV alanine. In addition, isolates 7.1 and 7.2 each contained a first position change causing the RNA to code for the PV amino acid valine instead of isoleucine at position 75. Isolates 8.1 and 8.2 each harbor two separate amino acid changes in addition to two silent third position changes. One of the third position changes is outside the hydrophobic domain in the codon for glutamic acid 53 in 3A, while the other silent mutation is the same third position change seen in the DIIS isolates 4.1 and 4.2 (leucine 73). The most striking feature of the D(I+II)S substitution is the consistent need for an alanine at position 65 as illustrated by the fact that all six isolates contained the same nucleotide change.

Sequence analysis of the DIIS isolates also revealed nucleotide mutations resulting in codon changes. Isolates 4.1 and 4.2 contained a first position change resulting in a reversion of isoleucine 75 to the PV valine, while isolates 6.1 and 6.2 each had a second position change resulting in the substitution of leucine 73 to the noncanonical amino acid serine. Curiously, isolates 4.1 and 4.2 each contained a third position change (adenosine to guanosine) in which the substituted amino acid (leucine) was maintained. Finally, the most interesting sequence results were obtained from DIIS isolates 5.1 and 5.2. The initial sequence analysis indicated that neither isolate contained any sequence changes throughout the entire 3AB coding sequence. The inability to find any nucleotide changes in these DIIS isolates, combined with the very low specific infectivity of the DIIS RNA, suggested the accumulation of a mutation(s) outside of the 3AB coding region. Indeed, a second site mutation was discovered when the complete genomes of both isolates 5.1 and 5.2 were sequenced. To this end, five overlapping RT-PCR fragments that span the entire poliovirus genome were sequenced. The results of this analysis are shown in Table 3. Some mutations outside of 3A were found in one isolate but not the other and represent nucleotide changes introduced by either the virus following the first round of plaque purification or polymerase errors during the subsequent genome amplification by RT-PCR. However, one mutation, C3930U, was found in both isolates and likely represents a virus-selected mutation that occurred early in the replication cycle of the virus that confers increased virus fitness in infected tissue-culture cells. This second site reversion results in a threonine-to-isoleucine change in amino acid number 33 of poliovirus protein 2B and provides novel genetic data that suggest 3A(B) and 2B may directly interact within the replication complex.

Analysis of RNA synthesis in virus-infected HeLa cells

To determine if the nucleotide sequence changes described above were responsible for increased viral RNA synthesis in infected cells, levels of virus-specific RNA accumulation were measured. Passage 1 virus stocks were used to infect HeLa cells at 37°C followed by the harvest of total cellular RNA at 0, 2, 4, and 6 h postinfection. The levels of PV-specific positive-strand RNA accumulation were measured by Northern blot analysis using a mixture of negative-strand oligonucleotide probes. As shown in Fig. 4, the kinetics of RNA accumulation for viruses containing the domain I substitution (DIS) were significantly delayed compared to those of wild-type virus (top panel, compare lanes 2, 6, and 10 to lane 14). The isolates containing the domain II substitutions (DIIS) contain multiple mutations and reversions within the HRV14 substituted sequences. These sequence variations do not produce dramatic differences in the kinetics of RNA accumulation (shown in the middle panel of Fig. 4) when compared to each other. However, all three DIIS isolates showed delayed rates of RNA accumulation compared to that of wild-type. Finally, for the mu-

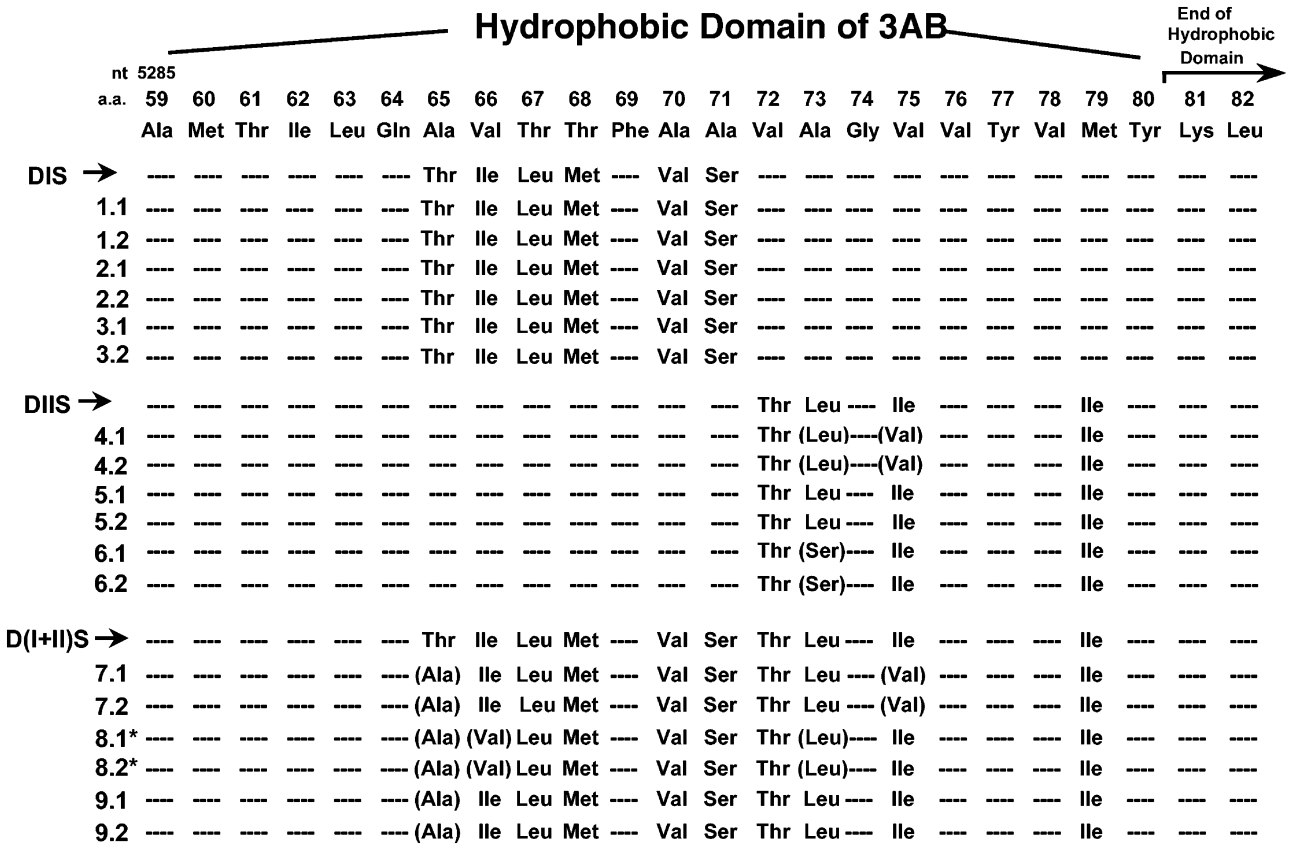

Fig. 3. Summary of the sequence analysis for each of the virus isolates. Shown on the top line is the amino acid sequence of the PV1 hydrophobic domain. Dashes indicate identity of the amino acid at that position with that of PV1. Amino acids different from those of PV1 are listed in the line corresponding to the sequence of each isolate. Amino acids in parentheses indicate that a nucleotide mutation was detected in that codon. The asterisks next to D(I+II)S isolates 8.1 and 8.2 indicate that the sequence analysis detected a third position change (A to G) in the codon for glutamic acid at position 53 of 3A.

tants containing the domain I+II substitutions [D(I+II)S], the kinetics of RNA accumulation were even more delayed compared to those of wild-type than any of the DIS or DIIS mutants. This can be seen by comparing lanes 2, 6, and 10 to lane 14 (bottom panel) as well as by observing the reduced levels of RNA present at 4 h after infection com-

Table 3
Summary of complete genomic sequencing of mutant PV1-3AB-DIIS 5.1 and 5.2

PCR fragment (PV1 nucleotides)	Nucleotide change	Codon change	Amino acid change	Comments
DIIS isolate 5.1				
Segment 1 (23–1023)	U761A	UCA to ACA	Ser to Thr	Not in isolate 5.2
Segment 2 (625–2879)	None			
Segment 3 (2615–4326)	**C3930U**	**ACC to AUC**	**Thr to Ile**	**Primary plaque mutation**
Segment 4 (3995–6074)	G4910A	GCC to ACC	Ala to Thr	Not in isolate 5.2
Segment 5 (5480–7432)	None			
DIIS isolate 5.2				
Segment 1 (nt 23–1023)	None			
Segment 2 (625–2879)	U2035C	CUU to CCU	Leu to Pro	Not in isolate 5.1
Segment 3 (2615–4326)	**C3930U**	**ACC to AUC**	**Thr to Ile**	**Primary plaque mutation**
	A3960G	AAC to AGC	Asn to Ser	Not in isolate 5.1
Segment 4 (3995–6074)	None			
Segment 5 (5480–7432)	None			

Note. Total RNA from cells infected with P1 stocks of isolates 5.1 and 5.2 were harvested and used to generate five overlapping RT-PCR fragments which were subsequently sequenced as described under Materials and methods. The U761A change corresponds to sequences in VP4, the U2035C change to sequences in VP3, the C3930U and A3960G changes to sequences in 2B, and the G4910A change to sequences in 2C. There are also a number of nucleotide changes common to both isolates that are in the full-length laboratory clone which are different from one of the published PV1 genome sequences (Kitamura et al., 1981; Racaniello and Baltimore, 1981). Those changes are U2308C, A3043G, C4174A, and C6261U. Bold notation indicates relevant second-site lesion in protein 2B coding region.

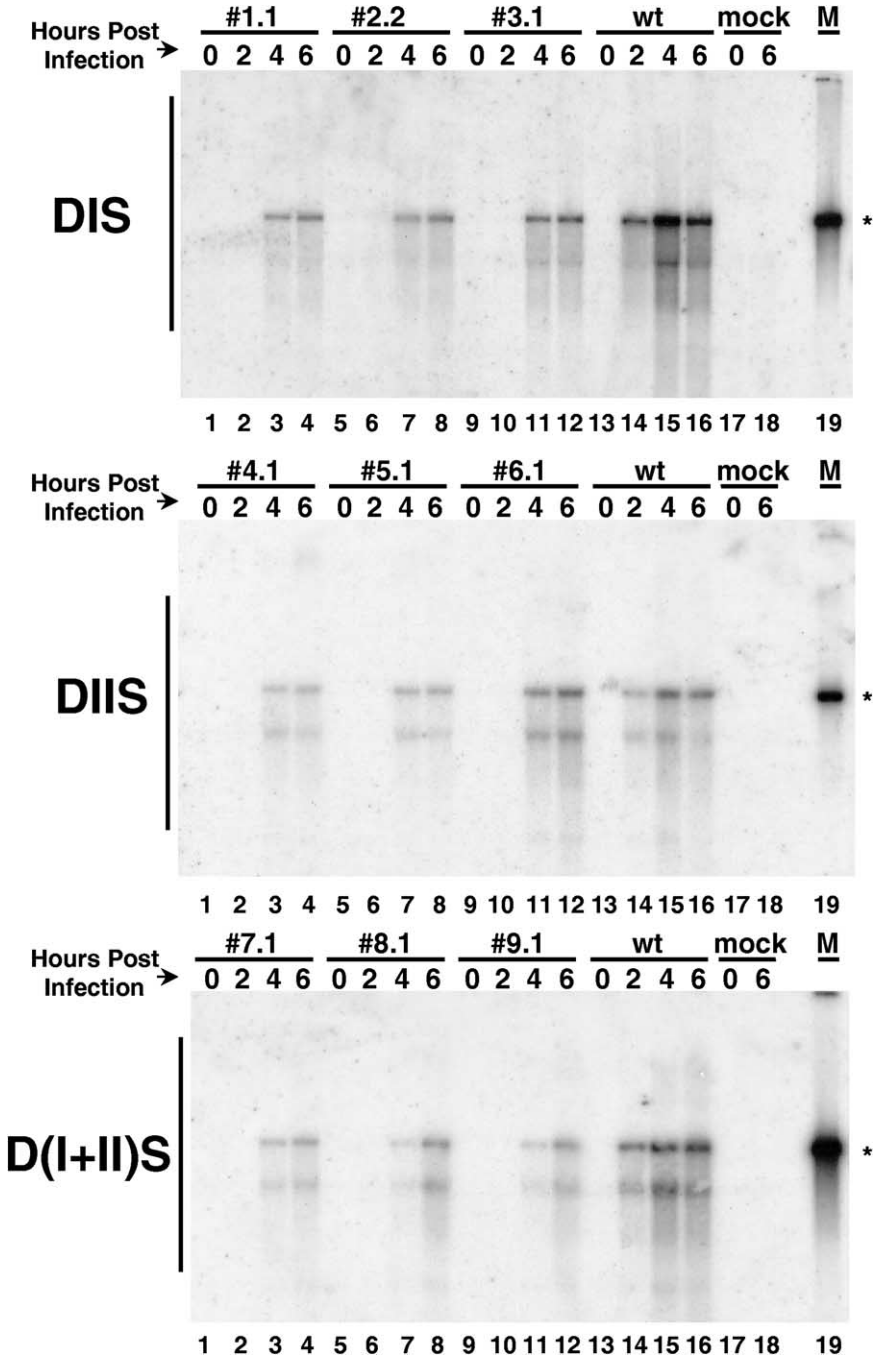

Fig. 4. Northern blot analysis of DIS, DIIS, and D(I+II)S mutants. HeLa suspension cells were infected at an m.o.i. of 10 using the passage 1 stock of the indicated virus isolate. At each of the indicated times after infection, total cellular RNA was harvested. Ten micrograms of total RNA was denatured and subjected to electrophoresis through a 1.1% agarose sodium phosphate gel. PV positive-strand RNA was detected using a mixture of three negative-strand oligonucleotides specific for the 2C coding sequences. As controls, total RNA from mock-treated HeLa cells was harvested at 0 and 6 h. The mobility of the 7.5 kb PV RNA is denoted by an asterisk in which the marker lane (lane 19) contained ~100 ng of PV virion RNA that was treated in parallel with the other RNAs. The top panel shows the analysis of DIS virus isolates, while the middle panel indicates that of DIIS. D(I+II)S isolates are shown in the bottom panel.

pared to that at 6 h. For all of the DIS and DIIS mutants, the RNA levels at 4 h postinfection were nearly identical to those at 6 h. By contrast, cells infected with the D(I+II)S mutants had significantly reduced RNA levels at 4 h after infection compared to those seen at 6 h.

Discussion

In this study, we have provided evidence for the partial conservation of function of the 3AB hydrophobic domain between members of the picornavirus entero- and rhinovirus

genera. Substitution of the amino half (domain I) of the poliovirus 3AB hydrophobic domain with the analogous sequences of HRV14 resulted in a mutant virus that has a significant RNA replication defect in addition to a slight defect in P3 protein processing. It is difficult to quantify the effects of the processing abnormality; however, it is noteworthy that the level of the viral 3D RNA polymerase is also reduced. Based upon the transfection data, substitution of the domain II or domain I+II sequences results in RNAs with reduced specific infectivities compared to those of wild-type or DIS RNAs. Substitution of the domain II sequences alone caused a severe replication defect, providing strong evidence for non-interchangeability of this domain. This result is somewhat surprising in that, relative to the domain I substitution, there are fewer amino acid changes present in the domain II substitution. In addition, the hydrophobicity of domain II is very well conserved, which might predict that the exchange of such sequences should be the least severe (Giachetti and Semler, 1990; Towner et al., 1996). Taken together, these experiments suggest that domain I of protein 3AB is more functionally conserved between rhinovirus and poliovirus than domain II. We suggest that sequences present in domain II could be involved in a higher order structure which is not tolerant of amino acid substitutions or that these sequences are involved directly or indirectly in the formation of a protein interface.

The possibility that the DIIS and D(I+II)S mutants harbor compensatory nucleotide changes outside of the 3A coding sequences was suggested by a number of observations. The in vitro replication data demonstrated that both DIIS and D(I+II)S display severe RNA replication defects, especially for D(I+II)S; yet the Northern blot analysis indicated that all of the corresponding mutant viruses have delayed, but much less debilitating RNA synthesis defects. Consistent with the increased fitness by the domain II containing mutants is that infection of HeLa monolayers at an m.o.i. of 10 required only 1.5 – 2 h longer to effect a similar level of CPE as wild-type virus infected at an identical m.o.i. The substantial primary defects in virus replication are also supported by the RNA transfection data in which virus plaques were seen only on monolayers receiving the highest amount of RNA (5 μg). This is in contrast to transfection of DIS RNA which resulted in similar numbers of plaques using 100 to 1000 times less RNA. The strongest evidence for the requirement of compensatory mutations comes from the analysis of DIIS isolates 5.1 and 5.2, which do not contain any nucleotide changes throughout the 3AB coding region. Complete genome sequence analysis revealed an identical change in both isolates that confers an amino acid substitution in poliovirus protein 2B. The overall significance of this finding is not yet clear, but it is tempting to speculate that this suggests a direct protein–protein interaction between 3AB (or 3A) and 2B within the replication complex and that such an interaction was perturbed by the substitution with the HRV14 sequences but restored by the compensatory change in 2B. The role of protein 2B in poliovirus RNA synthesis and replication complex formation has been previously demonstrated by a study in which three separate mutations in 2B conferred noncomplementable defects in RNA synthesis (Johnson and Sarnow, 1991). In addition, sequence motifs have been identified in coxsackievirus protein 2B that are important for viral RNA synthesis (Van Kuppeveld et al., 1995, 1996). Of particular interest is the report that recombinant coxsackievirus B3 (CVB3) genomes containing PV1/CVB3 chimeric 2B proteins expressed altered RNA replication phenotypes, depending upon which part of the 2B protein maintained the CVB3 sequences (Van Kuppeveld et al., 1997). The authors of this study also suggested that a sequence-specific interaction between amino acid sequences in the carboxy-terminal two-thirds of protein 2B and an unidentified viral protein may be required for at least one of the 2B RNA replication functions.

Third position codon changes seen in isolates 4.1/4.2 and 8.1/8.2 provide evidence for the intriguing possibility that some of the nucleotide substitutions may exert their effects at the level of RNA secondary structure and not necessarily at the amino acid level. In a report by de la Torre et al. (1992), the authors noted that a computer folding of a 150-nucleotide segment of the 3AB coding sequences (inclusive of the hydrophobic domain) predicts the formation of an extensive stem-loop structure with a ΔG° of -38.8. The idea that RNA secondary structure present within the coding sequences of positive-strand RNA viruses has functional significance for virus replication is not without precedent (e.g., Novak and Kirkegaard, 1994; Kim and Makino, 1995; Reynolds et al., 1995; Frolov and Schlesinger, 1996; McKnight and Lemon, 1998; Goodfellow et al., 2000; Paul et al., 2000; Yang et al., 2002). Furthermore, in the report by de la Torre and colleagues (1992), the authors directly examined the reversion frequencies of silent and codon-changing nucleotide substitutions within the hydrophobic domain of poliovirus protein 3AB. The authors discovered an apparent ability for the viral polymerase to simultaneously revert multiple third position (silent) changes (all transitions) despite the fact that the reversion of such nucleotides provided no measurable growth advantage in tissue culture over that of wild-type PV1. Thus, the replicase machinery is certainly able to simultaneously incorporate multiple nucleotide changes.

The data reported here suggest that there is a significant degree of conservation of protein structure/function between the entero- and rhinoviruses since virus was recovered from RNAs containing all three domain substitutions. For the most part, these substitutions did not produce 3AB structure or function exactly equivalent to that of wild-type PV1, since compensatory sequence changes were often required for efficient virus growth. Additionally, the results of this study warrant a more comprehensive analysis of the domain II containing virus isolates to determine by genome reconstruction if the discovered sequence reversions are

sufficient to relieve the replication and/or protein processing defects conferred by the HRV14 domain substitutions.

Materials and methods

Construction of HRV 14/PV1 hydrophobic domain substitutions

The cloning scheme for mutagenesis of the hydrophobic domain of poliovirus protein 3AB has been previously described (Giachetti and Semler, 1991; Giachetti et al., 1992), in which the genetically engineered *Pst*I site at PV nt 5301, the *Bgl*I site at PV nt 5321, and the genetically engineered *Hin*dIII site at PV nt 5351 were digested in pairwise combinations to introduce annealed oligo cassettes encoding the corresponding HRV14 sequences. The cassette used for the domain I substitution was the oligo 5′-GACTATTCTGATGTTTGTCAGTG-3′ annealed to the oligo 5′-CACAGACAAACATCAGAATAGTCTGCA-3′; the cassette used for the domain II substitution was the oligo 5′-CAACATTAGGTATTGTTTATGTCATTTATA-3′ annealed to the oligo 5′-AGCTTATAAATGACATAAACAATACCTAATGTTG-3′; and finally, the cassette for the domain I+II substitution was made by annealing the oligonucleotides 5′-GACTATTCTGATGTTTGTCAGTACATTAGGTATTGTTTATGTCATTTATA-3′ and 5′-AGCTTATAAATGACATAAACAATCCATAATGTACTGACAAACATCAGAATAGTCTGCA-3′. Note that for the domain I and domain II substitutions, the *Bgl*I site was blunt-ended using T4 DNA polymerase prior to ligation.

RNA transcription, RNA transfection, and plaque assays

RNA transcriptions were performed essentially as described (Towner et al., 1996) with the following changes: (a) the plasmids were linearized with *Eco*RI and (b) RNAs used for transfections were not extracted with phenol/chloroform or ethanol precipitated but were used in crude form following the 37°C incubation. The RNA transfections were performed using DEAE-dextran following the procedures outlined in Charini et al. (1991). Plaque assays were performed in 60-mm-diameter dishes under semisolid media at 33 or 37°C for the indicated amount of time. Dishes were then overlaid with 10% trichloroacetic acid for 45 min, followed by staining with crystal violet prior to plaque counting.

Analysis of RNA by Northern blot

Total cytoplasmic RNA was harvested by the method of Favaloro et al. (1980) with previously described modifications (Roehl et al., 1993). Northern blot analysis was performed by resolving 10 μg of glyoxylated total cellular RNA on a 1.1% agarose gel in 10 mM sodium phosphate buffer (McMaster and Carmichael, 1977) and transferring the RNA to a GeneScreen membrane (DuPont). The blot was then probed with a mixture of the 5′ end-labeled, negative-strand oligonucleotide probes JT2C4864− (complementary to PV nt 4845–4864), JT2C4588− (complementary to PV nt 4569–4588), and JT3A5130− (complementary to PV nt 5110–5130), which are specific for the sequences of the poliovirus 2C region.

Sequencing of recovered viruses

Sequencing of viral RNA encoding the carboxy-terminal half of 3A was performed by first amplifying the entire 3AB sequence by RT-PCR (50 μl total volume) using the primers CGS1− (anneals to sequences in VPg; Giachetti and Semler, 1991) and JT2C5060+ (anneals to PV nt 5060–5080). A portion (3 μl) of each RT-PCR reaction was then used in an additional 50 μl PCR reaction in which only the primer JT2C5060+ was provided, such that a positive-sense single-stranded DNA was asymmetrically amplified. Following asymmetric PCR, each reaction was passed through a Sephadex G-50 column to remove unincorporated oligonucleotides and dNTPs. Approximately half of each reaction was then used for dideoxy sequencing using the primer CGS1− and phage T7 DNA polymerase. Sequencing of the complete genomes of DIIS isolates 5.1 and 5.2 was carried out by amplifying by RT-PCR five overlapping domains spanning the entire poliovirus genome (see Table 3 for the nucleotide numbers of each RT-PCR fragment). Each RT-PCR product was gel purified prior to automated sequence analysis by the UC Irvine Automated DNA Sequencing Core Facility or Biotech Diagnostic, LLC (Laguna Niguel, CA).

Coupled in vitro translation/replication assays

Assays were performed essentially as described in Todd et al. (1997), in which the HeLa ribosomal salt wash (RSW) preparation was performed as described by Brown and Ehrenfeld (1979), and the HeLa S-10 extract preparation was performed as described by Barton et al. (1995). Minor differences or new modifications in either the extract preparation or the translation/replication assay are outlined as follows: (a) HeLa cells were resuspended in hypotonic buffer in a volume equal to 120% of that of the cell pellet volume; (b) each replication reaction contained 51% (v/v) HeLa S-10, 18% (v/v) RSW preparation, 21% (v/v) DEPC-treated H_2O, and 10% (v/v) of a 10× mix containing (at 10×) 10 mM ATP, 2.5 mM GTP, 2.5 mM UTP (no CTP was added), 600 mM potassium acetate, 300 mM creatine phosphate, 4 mg/ml creatine kinase, and 155 mM HEPES-KOH (pH 7.4); (c) reaction mixtures (50 μl), programmed with in vitro transcribed RNA (9.6 nM), were divided into portions containing 10 and 40 μl. The 10-μl portions [containing an additional 10.5 μCi [^{35}S]methionine (Amersham) at >1000 Ci/mmol] were used for analysis of translation and protein processing, while the 40-μl reaction was used

for analysis of RNA synthesis. All reactions were incubated for 6 h at 30°C, at which time the translation reactions were diluted in Laemmli sample buffer (Laemmli, 1970), boiled, and subjected to SDS–polyacrylamide gel electrophoresis on a 12.5% gel. The replication reactions were subjected to centrifugation at 15,000 *g* for 15 min at 4°C and subsequently resuspended in 5 μl of buffer (50 mM HEPES pH 8.0, 3 mM $MgCl_2$, 10 mM DTT, 0.5 mM each of ATP, GTP, and UTP) containing 25 μCi of [α-^{32}P]CTP and incubated for an additional hour at 37°C. Total RNA was then extracted from each sample using phenol:chloroform (1:1), ethanol-precipitated, and resuspended in DEPC-treated H_2O. Total RNA was then subjected to gel electrophoresis on a 1.1% agarose TBE gel containing ethidium bromide. In an adjacent lane, ~500 ng of vRNA was loaded as a marker to visualize the mobility of single-stranded poliovirus RNA.

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